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ALTERATIONS IN HEPATIC AND AORTIC PHOSPHOLIPASE-C COUPLED RECEPTORS AND SIGNAL TRANSDUCTION IN RAT INTRAPERITONEAL SEPSIS

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INTRODUCTION

Sepsis, septic shock and endotoxemia are characterized by a number of metabolic and cardiovascular alterations. The metabolic changes include altered glucose utilization and a diminished ability of catecholamine and other glucogenic agents to mobilize stored glucose (Clemens et al, 1984; Spitzer, JJ this volume). Cardiovascular changes include a diminished systemic vascular resistance (SVR), normal or elevated cardiac output (CO) and an attenuated ability of exogenous pressor agents to raise blood pressure (see Chernow and Roth 1986a; 1986b for review). Despite years of intensive research, the precise molecular and cellular mechanisms for these derrangements have, until recently, remained obscure.

One common idea which links these diverse metabolic and cardiovascular changes is the concept that altered receptor-mediated signal transduction may serve as a mechanism (Chernow and Roth 1986a; 1986b). This is a particularly powerful hypothesis since it can explain both the changes in hepatic glucose mobilization as well as the diminished SVR and response to exogenously administered catecholamines seen in septic shock. This is because the signal transduction mechanism for glucose mobilization in the liver as well as vascular contraction are quite

similar (Fig 1).

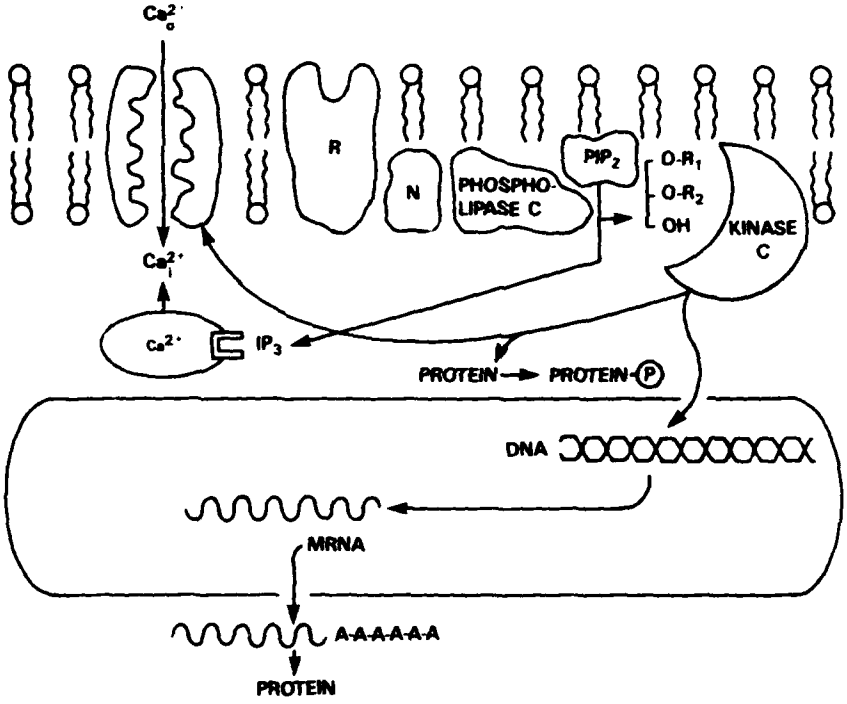


Figure 1. A model for signal transduction involving phospholipase-C coupled receptors. According to the scheme above (*modified from* Roth and Chuang, 1987), following binding of a ligand to its specific receptor (R), a phosphoinositide-specific phospholipase C is activated which selectively cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) to yield diacylglycerol (DAG) and inositol trisphosphate (IP3). The DAG activates protein kinase C (PKC) while the IP3 mobilizes intracellular calcium. Certain receptors (e.g. the aortic alphal-adrenergic receptor) may alter ion flux directly through Ca^{++} channels by an unknown mechanism. PKC may also augment Ca^{++} flux through this channel (Litten et al, 1987). PKC may phosphorylate other cellular proteins; there is evidence accumulating which suggests that PKC can affect gene transcription through direct interactions with DNA binding proteins. Oncogene mRNA, for example, has been shown to be elevated following PKC activation in many cell types.

Alphal-adrenergic and vasopressin receptors in both liver and aorta are coupled to a phosphoinositide (PI)-specific phospholipase C. In the liver, activation of phosphorylase a is dependent upon activation of PI hydrolysis, while in aorta vascular contraction is caused, in part, by PI breakdown (Nakaki et al, 1986; Roth et al, 1986). In aorta, in contrast to liver, voltage-gated and receptor-operated calcium channels are important for mobilization of extracellular calcium. We have previously proposed that the phasic and tonic components of contraction are mediated by PI hydrolysis and by calcium channel activation, respectively (Nakaki et al, 1985). In aorta, prostaglandins (Suba et al, 1987) and serotonergic agents (Roth et al, 1984; 1986) also transduce their signals by activation of PI hydrolysis.

Other workers have demonstrated, using perfused liver preparations, that the alphal-adrenergic receptor stimulated mobilization of glucose is attenuated in various rat models of sepsis (Clemens et al, 1984; Chaudry et al, 1986). In a similar fashion, Pomerantz et al (1982) and Wakayabashi et al (1987) showed an attenuated ability of norepinephrine (NE) to induce rat aortic contraction in their model of endotoxemia. Because these changes implied that the alphal-adrenergic receptor signal transduction system might be altered in sepsis and endotoxemia, we began a systematic study of this important system using various models of sepsis and endotoxemia.

RESULTS

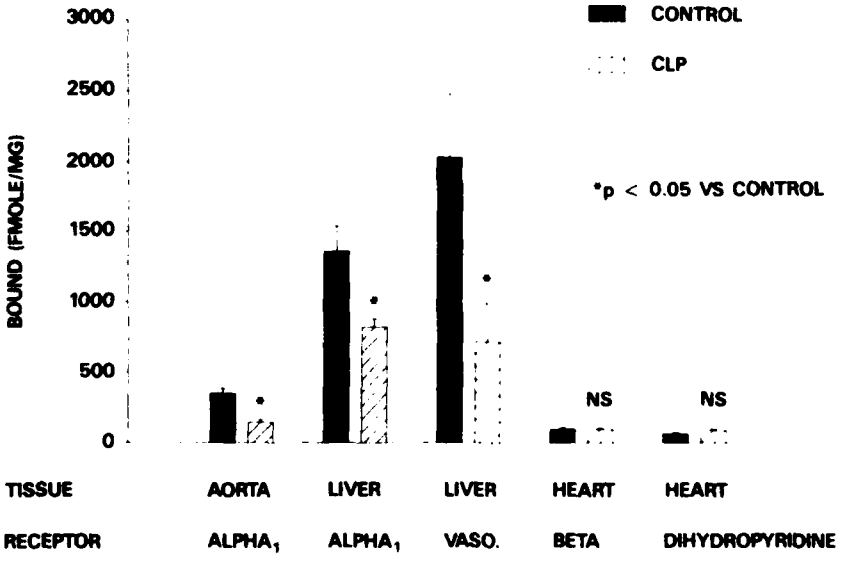
Hepatic Phospholipase-C Coupled Receptors are Altered in Sepsis and Endotoxemia

We first elected to study hepatic receptors since liver is a rich source of PI-coupled receptors and because hepatic plasma membranes are easily obtained in a highly purified condition. We isolated hepatic plasma membranes by a modification of the Prpic et al (1984) method (McMillan et al, 1986). The inclusion of protease inhibitors was necessary to obtain fully coupled receptors. We also utilized the cecal-ligation and puncture model of Wichterman et al (1980) as previously described (McMillan et al, 1986)

As is seen in Fig 2, alphal-adrenergic receptors were

decreased by nearly 50% when purified plasma membranes were studied. Also, [Arg^8-vasopressin receptors (measured with [^3H]-[Arg^8]-vasopressin were also diminished to a similar extent. As we previously showed (McMillan et al, 1986), guanine-nucleotide coupling was unchanged when membranes harvested from septic rats were compared with sham operated animals. These results suggested that although the number of alphal-adrenergic receptors was diminished, coupling was unaffected. Similar results have recently been published using a chronic endotoxin infusion model (Roth and Spitzer, 1987). In all of the studies, no changes in receptor affinity were noted, suggesting that these changes represent alterations in the numbers of receptor molecules.

We wondered whether other adrenergic receptors might be altered in liver as well and chose to study beta-adrenergic receptors using [^{125}I]-iodocyanopindolol. No changes in beta-adrenergic receptors were noted (Fig 2). We also investigated cardiac tissue since changes in cardiac contractility have been noted in numerous sepsis models and in humans (Chernow and Roth, 1986a). No changes in beta-adrenergic or dihydropyridine receptors were noted when purified microsomes obtained from septic rat hearts were assayed (Fig 2).



We next wondered whether these receptor changes could be a result of proteolytic degradation of the receptor recognition sites. We chose to focus on alphal-adrenergic receptors since these have been among the most intensely studied. Using [^{3}H]-phenoxybenzamine (POB) to cross-link the alphal-adrenergic receptors we found no changes in the apparant molecular weights (Mr) when receptors from sham-operated livers were compared with those from CLP rats Three major peaks of POB binding were seen at 146 Kda, 71 Kda and 56 Kda. The 146 peak probably represents unreduced dimer, while the 71 Kda protein represents the native receptor (Venter et al, 1984). The 56 Kda peak represents the major proteolytic fragement of the receptor (Venter et al, 1984). If proteolysis occured, little 71 Kda peak would be present and numerous smaller molecular weight peaks would be found (Venter et al, 1984). These findings demonstrated that little proteolytic degradation of hepatic alphal-adrenergic receptors occurs during intraperitoneal sepsis.

Alterations in aortic alphal-adrenergic receptor mediated signal transduction in sepsis.

We next elected to study rat aorta to determine whether similar or different changes were found in vascular tissue. We first measured alphal-adrenergic receptors using [^{125}I]-hydroxy-ethyl-aminotetralone (HEAT) which specifically labels alphal-adrenergic receptors in many tissues. Preliminary studies demonstrated specific labelling of alphal-receptors.

Figure 2 shows that the alphal-adrenergic receptors in purified microsomes obtained from aortas from control and septic rats were diminished in number. No changes in affinity were found (not shown). We previously showed that aortic alphal-adrenergic receptors transduced their signals, in part, via activation of PI hydrolysis (Legan et al, 1985).

Figure 2. Alterations in hepatic and aortic receptors in rat intraperitoneal sepsis. The figure shows the maximum number of binding sites on the Y-axis (Bmax) for control (solid bars) and septic (cross-hatched bars) rats for various organs. *$p<0.05$ vs sham-operated controls. The data represent mean ± sem of 5-10 individual experiments.

Figure 3 demonstrates that NE-induce PI breakdown, as measured by [^{3}H]-inositol monophosphate (IP) accumulation was attenuated in aortas from septic rats. The figure also shows a decrease in basal IP production as well, which implies that synthesis of PI might be altered in sepsis.

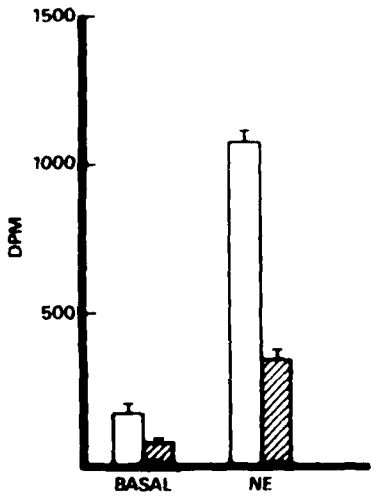


Figure 3. Alterations in NE-stimulated PI hydrolysis in sepsis. Shown are results from 6 individual experiments. The changes in basal and stimulated IP accumulation are significant at the 0.05 level.

The decrements of basal [^{3}H]-IP accumulation suggested to us that selective changes in PI synthesis might be evident in sepsis. To test this hypothesis we labelled phosphoinositides with [^{32}P]-orthophosphate under conditions in which equilibrium labelling occured. Figure 4 shows that only the synthesis of phosphatidylinositol-4,5-bisphosphate (PIP2) was diminished in sepsis. No changes in phosphatidylinositol-4-phosphate, phosphatidyl-inositol or phosphatidic acid were noted (Figure 4). These results imply that not only is the receptor-stimulated PI breakdown attenuated in sepsis, but that synthesis of PIP2 is also selectively attenuated.

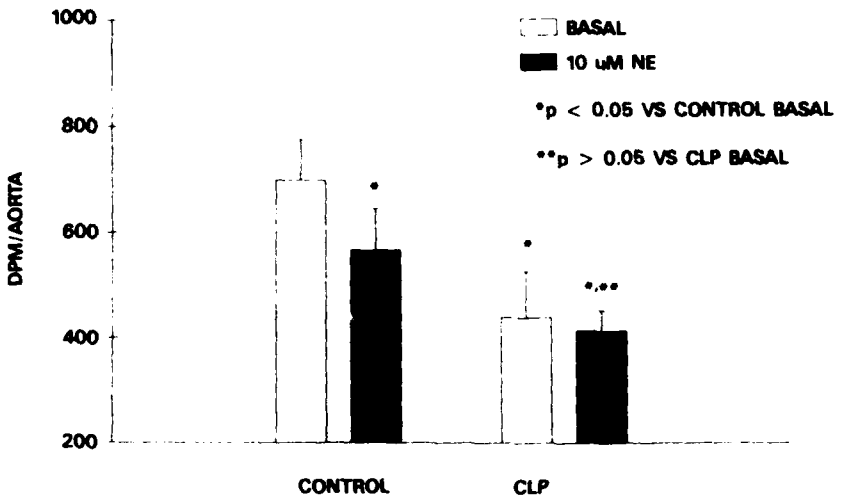


Figure 4. Alterations in phosphoinositide synthesis in aorta during intraperitoneal sepsis. Rat aortas were incubated with [^{32}P]-orthophosphate under conditions in which equilibrium labelling was obtained. After 30 min of labelling, 10 μM NE was added; 30 sec later the reaction was terminated and Phospholipids were separated on oxalate-precoated high performance thin layer chromatography plates according to Jolles et al (1981). Labelled phospholipids were visualized by X-RAY film, scrapped and counted by liquid scintillation. Data represent mean ± sem of 4 individual experiments. (*p 0.05 vs control). No changes in basal levels of PI, PIP or PA were noted (not shown).

We next determined whether the hydrolysis of PIP2 was altered to a similar extent in aortas from septic rats. This is important since PIP2 is thought to be the initial substrate of the nucleotide regulated phospholipase C in rat aorta (Roth and Chuang, 1987; Roth, 1987).

Figure 4 also shows that the NE-stimulated PIP2 breakdown was significantly decreased in aortas from septic rats. Following PIP2 breakdown, in many systems, calcium is mobilized through the sarcoplasmic reticulum. In aorta, calcium may also be mobilized through calcium channels in the plasmalemma. Figures 5 and 6 show that the NE-stimulated Ca++ efflux (via the sarcolemma through

IP3 production) and influx (through calcium channels) were both decreased in sepsis.

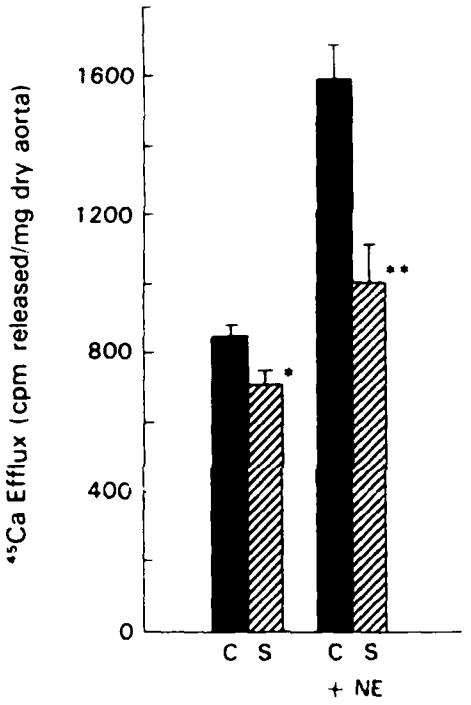


Figure 5. Alterations in calcium efflux in sepsis. Shown are results obtained from 12-16 septic rats. 10 μM NE was used for this and all subsequent experiments.

One other product of PIP2 hydrolysis, DAG, has been shown to activate PKC (Nishizuka, 1984). It is conceivable that PKC might be altered as well during sepsis. We therefore measured PKC using [^{3}H]-phorbol-12,13-dibutyrate (PDBu) according to a previously described protocol (Sando et al, 1984). No changes in PDBu binding sites were noted suggesting that

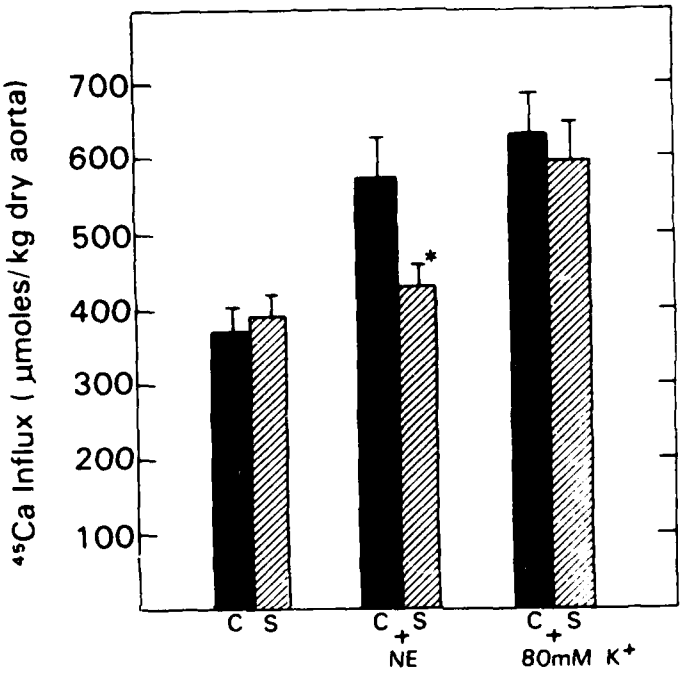


Figure 6. Alterations in calcium influx in sepsis. Shown are results obtained from 12-16 aortas from control and septic rats. As is seen only the NE-stimulated calcium influx was significantly altered while K+-stimulated influx was unchanged.

the absolute levels of PKC in rat aorta are unchanged during sepsis (not shown). This is contrast to a recent study by Wakabayshi et al (1988) who showed that the PDBU-induced contraction of rat aorta after endotoxin adminstration is diminished.

Other studies have demonstrated an attenuated ability of NE to induce protein phosphorylation (Carcillo et al, 1987). We found selective decreases in the ability of NE to phosphorylate a 25 KDa phosphoprotein in rat aorta during sepsis. We suggest that, ultimately, altered protein phosphorylation could mediate the effects of the diminished signal transduction on contraction of rat aorta.

DISCUSSION

In this paper we report that hepatic and aortic phospholipase-C coupled receptors are decreased in number during rat intraperitoneal sepsis; no changes in agonist or antagonist affinity or receptor-G protein coupling were identified. We also discovered that the alphal-adrenergic receptor mediated signal transduction system in rat aorta was modified as well during intraperitoneal sepsis. Thus, each step of the adrenergic signal cascade (PI hydrolysis, Ca++ mobilization and protein phosphorylation) was diminished. These results suggest that signal transduction involving aortic alphal-adrenergic receptors is attenuated during intraperitoneal sepsis.

Previous investigators (Fink et al, 1984) previously showed that the ability of NE and angiotensin II (AII) to raise the mean arterial pressure (MAP) was decreased during intraperitoneal sepsis. Other investigators (Scheller et al 1985) found that the ability of NE, AII and Vaso to increase MAP during endotoxemia in the rat was similarly altered. We as well discovered that phenylephrine's capacity to augment the MAP was blunted during intraperitoneal sepsis (Disimone and Roth, unpublished observation). All these results imply that fundamental adaptations occur during sepsis which affect the ability of pressor agents to maintain the blood pressure. Keywords: Vasoconstriction [illegible]

The Role of Prostanoids in the Altered Signal Transduciton During Sepsis

The Fink et al (1984) study found that the diminshed pressor activity of NE and AII could be ameliorated by pretreatement with indomethacin which inhibits prostanoid production. These authors thus suggested that local or systemic production of prostanoids might be responsible for the alterations in sensitivity they found. One might suggest that prostanoids alter the ability of alphal-adrenergic agents to augment second messenger production. We tested this hypothesis by two types of experiments: (1) we studied the effects of prostanoids on aortic PI metabolism alone and (2) we determined whether

prostanoids might lessen the ability of NE to augment PI hydrolysis. We found (Suba et al, 1987) that, contrary to our expectations, the various prostanoids we studied all activated PI hydrolysis in rat aorta and that they did not diminish the ability of NE to aument PI hydrolysis (Suba et al, 1987).

In a similar fashion, Pomerantz et al (1982) studied the isolated rat aorta during endotoxemia. They found a lessened capability of NE to induce contraction. Pretreatment of rats in vivo with indomethacin or treatment of aortic rings in vitro did not ameliorate the contractile deficit they discovered. These results all indicate that locally or systemically produced prostanoids cannot account for the changes in signal transduction we observed in aorta. Wakayabashi et al (1987) found that the alterations did not require the presence of endothelium in constrast to results obtained by McKenna (this volume).

Potential Causes for the Perturbed Signal Transduction During Sepsis

What then accounts for the changed signal transduction which occurs during intraperitoneal sepsis? Several possibilities are evident: (1) down-regulation due to excessive stimulation by calcium and endogenous ligands; (2) direct and indirect effects of lymphokines; and (3) direct effects of lipopolysaccharide (LPS; endotoxin). These potential causes will be examined indivdually and evidence for and against them will be presented.

Several investigators (Jones et al, 1982; Schuler et al, 1985) have demonstrated that during endotoxemia and early sepsis the levels of catecholamines and other vasoactive agents (e.g. Vaso and AII) are strikingly elevated. Prolongued elevation of these agents can be expected to induce down-regulation of their respective receptors. In fact, Rosenbaum et al (1986) showed that chronic elevation of plasma catecholamines by exogenous administration and pheochromocytomas caused a diminshed ability of NE to augment PI hydrolysis in rat aorta. Thus, the attenuated alpha1-adrenergic receptor mediated signal transduction we found during sepsis could be due to elevated circulating catecholamines. The changes in basal PIP2 synthesis, though, are not accounted for by this

mechanism.

Other investigators found that in the liver there was an elevated basal level of Ca++. Sayeed and Maitra (1987) showed that not only was basal Ca++ elevated during endotoxemia, but that the ability of NE to augment Ca++ was similarly blunted. Indeed, for many years investigators have speculated that a raised intracellular Ca++ occured during sepsis and endotoxemia and that this was an important factor for the concomittant cellular toxicity. It is conceivable that the elevated Ca++, by an as yet undefined mechanism, induces a compensatory down-regulation of the Ca++-mobilizing receptors and transducing systems. This could, potentially, occur via a number of mechanisms: (1) activation of a calcium-specific protease (e.g. calpain); (2) activation of protein kinase C (see McMillan et al, 1986 and Roth et al, 1986 for evidence implicating protein kinase C in receptor desensitization) and (3) activation of other Ca++-sensitive enzymes (e.g. calmodulin, Ca+-ATPase etc). At the present time there is no evidence implicating any of these possible mechanisms.

It is important to realize in this regard, though, that not all investigators have noted elevated resting Ca++ levels during endotoxemia and sepsis. For example, Deaucic and Spitzer (1986) showed that during both acute and chronic endotoxemia and intraperitoneal sepsis resting levels of Ca++ were actually diminished. These authors also found, which Maitra and Sayeed reproduced (1987), that the capability of NE and Vaso as well as IP3 to augment intracellular Ca++ concentrations was markedly diminshed. Therefor, the evidence for a "Ca++-poisoning" during endotoxemia and sepsis is equivocal. To conclude, then, the hypothesis that excessive stimulation due to either Ca++ or endogenous hormones leads to down-regulation of the signal transduction systems, although seductive, is at present incompletely tested.

The role of lymphokines in the receptor desensitization noted during sepsis is speculative, since few direct studies have been attempted. Most recently McKenna (this volume) found that incubation of aortic rings with Interleukin I (IL1) and Tumor Necrosis Factor (TNF) decreased the ability of NE to augment contraction.

However, this author was unable to show that PI hydrolysis was altered to an extent similar to that found during intraperitoneal sepsis in both liver (see Spitzer et al 1987) and aorta. Thus, although, IL1 and TNF may both cause alterations in aortic contractility which are phenomenologically similar to those found during sepsis, mechanistically they appear quite distinct.

Finally, we come to the role of LPS in inducing these changes. Direct incubation of aortic rings for extended periods of time with LPS (Nakaki and Roth, unpublished observation) does not alter basal or NE-stimulated PI hydrolysis. It is possible, however, that LPS in vivo is presented to the aortic smooth muscle cells in a way which is differenct from that in vitro.

LPS was found (Weightmann and Raetz, 1984; Raetz this volume) to activate protein kinase C in vitro. Activation of protein kinase C by phorbol esters induces many of the changes in signal transduction we found during sepsis (see McMillan et al, 1986; Roth et al, 1986; Roth and Chuang, 1987). The mechanism of activation of protein kinase C by LPS and by phorbol esters, though, is quite different. In the case of phorbol esters, they appear to activate the enzyme by lowering its threshold (decrease Km) for Ca++ so that the enzyme is fully activated by resting Ca++ levels. LPS, on the other hand, does not affect the ability of Ca++ to activate the enzyme; instead LPS may substitute for phospholipids like phosphatidylserine to activate the enzyme. Exactly how LPS might activate PKC during sepsis is unknown, since more than adequate amounts of phospholipids are present in the cellular mileau for full activation.

There is also evidence that LPS may activate PI hydrolysis and PKC via interaction with a specific receptor molecule on certain cells (Ogmunsdotter and Weir, 1979; Rosoff and Cantley, 1985; Prpic et al, 1987). Whether such a mechanism is important in liver and aorta is not known. In preliminary experiments, though, we could find no evidence for a direct action of LPS on aortic PI metabolism (Nakaki and Roth, unpublished observation).

Therefore, the idea that LPS, via a direct interation with PKC, might cause the blunting in receptor-mediated signal transduction seen during sepsis is attractive but, at present, incompletely tested.

What then is the cause of the alterations in signal transduction seen during sepsis? Some of the best evidence favors the idea of a compensatory down-regulation due to excessive stimulation by endogenous ligands (e.g. catecholamines and hormones). This idea is potentially testible by pre-treating experimental animals with receptor antagonists which should blunt the effects of intense stimulation due to circulating agents. It must be noted, though, that not all receptors are down-regulated during sepsis. We could not find evidence for beta-adrenergic receptor alteration in heart or liver (Fig 2); such changes might be expected if down-regulation was a general mechanism of tachyphylaxis during sepsis.

Future Directions

As is evident from the preceeding discussion, the precise reason for alterations in signal transduction is unknown. Much work must be done in the future to sort out all the potential causes and mechanisms. One idea which has not been tested, but which shows great promise for the future, is the idea that the changes noted are due to perturbations in mRNA and protein synthesis.

It is now becoming clear (see Fig 1) that in addition to the immediate effects of receptor occupancy (e.g. receptor binding and activation of second messenger systems) there are subacute and chronic effects which have only recently been possible to study. In particular, receptor binding appears, in many systems, to induce transcription of genes. The most completely studied have been oncogenes like c-FOS and c-Myc (see Carcillo and Hough, this volume for review). In this regard, Carcillo and Hough (this volume) discovered that incubation of aortic rings with either LPS or NE induces c-myc expression. This induction occurs as early as 2 hr after the agent is applied. Thus, LPS and NE can alter levels of mRNA for specific genes.

With the availability of full-length probes to the alphal-adrenergic receptor we should soon be able to test the idea that receptor mRNA synthesis is altered during sepsis. If such changes are discovered, they could have the potential for discovering the locus of action of LPS on a molecular level within the cell.

It is also evident that the final common denominator in transducing phospholipase-C coupled receptor messages, i.e. PKC, exists as multiple forms. In fact as many as 8 distinct isozymes might exist in rat brain alone. We have recently purified PKC to homogeneity (Roth et al, submitted) and have prepared antibodies to 4 of these isozymes. Additionally, we have prepared synthetic oligodeoxynucleotide probes to each of these isozymes and have developed techniques to quantify the mRNA levels for each isozyme (Roth and Iadarola, submitted for publication). Since levels of PKC apparantly change during sepsis and chronic endotoxemia (see Hermiller et al, this volume), it is conceivable that LPS alters the transcription of individual PKC isozymes. Future work with these specific probes should be forthcoming. These studes as well have the potential to elucidate the effects of LPS at the level of gene transcription.

CONCLUSIONS

As is evident, there is now much evidence implicating phospholipase-C coupled receptors in the pathophysiology of sepsis and endotoxemia. Specifically, desensitization at each step of the receptor-transducer cascade is seen in aorta as well as in liver (see JA Spitzer this volume). The mechanism of these changes is at present unknown. Future studies utilizing the techniques of modern molecular biology might shed light on these intriguing phenomena.

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DISCUSSION

Dr. Mark Clemens (Johns Hopkins Hospital): You have presented some rather intriguing results I think very strongly supporting your scheme in the IP_3 mediated signal trasnduction in sepsis. With respect, though, to your contention that other signal transduction systems seem to be relatively intact, for instance Dr. Leu has shown several years ago that beta receptors in dog heart are decreased during endotoxin infusion or species? Or is this just an aberrant case?

Dr. Roth: We haven't published the data yet, but we as well cannot find any changes in beta adrenergic receptors in dog heart. In particular, we wondered if perhaps it was a change in internalization and perhaps we were measuring all the receptors instead of just the surface receptors.

So we did the studies with hydrophobic and hydrophilic ligands and we still cannot find any change in beta receptors. As Steve Jones, though, has shown at Loyola, if you look at rats at earlier stages, predeath stages, at least in our hands we cannot find any changes, and particularly in the chronic endotoxemia model in which the rats don't die, and in the Parillo model in which the dogs don't die. We just could not find any changes in the beta receptors.

Dr. Chuang: Bryan, I would like to ask you whether the attenuation you see after septic shock in the alpha-1 and the media response is due to activation of protein kinase C?

Dr. Roth: Christian Raetz is here, and he earlier showed that endotoxin activates protein kinase C. There really is a lot of evidence suggesting that activation of protein kinase C can cause desensitization. At the present time, we have no direct evidence implicating this, but this is a very attractive hypothesis at present for the tachyphylaxis that we see.

Dr. Raetz: Basically we just looked at it in vitro under the standard protein kinase C assay conditions and we found that endotoxin or many of the lipid A precursors which are acidic phospholipids basically substitute for phosphatidylserine. Now whether that is physiologically relevant or not, I don't think our data really addresses, but that is the observation.